1 **Feeding behavior in relation to spittlebug transmission of *Xylella fastidiosa***

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Abstract

Here we provide the first insights into the transmission dynamics of the bacterium *Xylella fastidiosa* by the meadow spittlebug *Philaenus spumarius*, gathered through DC-EPG (Electrical Penetration Graph)-assisted transmission tests and comparative observations of the probing and feeding behavior of infective versus non-infective vectors on healthy olive plants. Bacterial cells binding to *P. spumarius*' foregut occurred at a very low rate and in a time as short as 15 minutes spent by the insect in xylem ingestion or activities interspersed with xylem ingestion (interruption during xylem ingestion and resting). *P. spumarius* inoculation of bacterial cells into the xylem was exclusively associated with an early (ca. 2 to 7 minutes after the onset of the first probe) and occasional behavior, provisionally termed waveform Xe, presumably related to egestion regulated by pre-cibarial valve fluttering. Infective spittlebugs compared to non-infective ones exhibited: i) longer non-probing and shorter xylem ingestion; ii) longer duration of single non-probing events; iii) fewer sustained ingestions (ingestion longer than 10min) and interruptions of xylem activity (N); iv) longer time required to perform the first probe. These observations suggest difficulties in feeding of infective *P. spumarius* probably caused by the presence of *X. fastidiosa* within the foregut. Overall, our data indicate that likely short-time -few minutes- is required for *X. fastidiosa* transmission by *P. spumarius*, thus vector control strategies should aim at preventing spittlebug access to the host plant. Furthermore, our findings represent an important contribution for further research on the disruption of spittlebug-bacterium interactions.

Keywords: *Philaenus spumarius*; EPG; olive; oleander; transmission dynamic; fastidious bacterium

34 **Key message**

35 - Here we provide the first insights into the transmission dynamics of the bacterium
36 *Xylella fastidiosa* by its main European vector, the spittlebug *Philaenus spumarius*.
37 - Acquisition occurs at a very low rate during the first minutes the insect is ingesting the
38 xylem sap. Inoculation is likely related to an occasional behavior that occurs early in the
39 probe, possibly egestion regulated by pre-cibarial valve fluttering. Infective spittlebugs
40 exhibited feeding difficulties possibly caused by the presence of the bacterium within
41 the foregut.
42 - Given the short-time required for *X. fastidiosa* transmission, vector control strategies
43 should aim at preventing spittlebug access to the host plant.

44

Introduction

Since the first report of a grapevine disease (Pierce 1892) later found to be caused by a vector-borne microorganism successively identified as a bacterium (Davis et al. 1978), named *Xylella fastidiosa* (Wells et al. 1987), research has clarified many aspects of bacterium-vector-host plant interactions. Nevertheless, an essential question still remains unanswered: what are the vector behaviors necessary for bacterial transmission to plants (Almeida 2016)? *X. fastidiosa* is a xylem-limited bacterium, whose natural spread relies on insects specialized for feeding on xylem sap (Houston et al. 1947; Frazier 1965). Therefore, it is assumed the vector should access xylem vessels to acquire the bacterium as well as to inoculate it (Houston et al. 1947); however, the exact behaviors, or sequence of behaviors resulting in transmission are unknown. Vector acquisition efficiency is a direct function of vector access period to the source plant, and of the bacterial population inside the infected tissue (Purcell and Finlay 1979; Hill and Purcell 1997). Following acquisition, *X. fastidiosa* cells bind to the vector foregut, putatively to the portion of the precibarium proximal to the cibarium, behind the pre-cibarial valve (Almeida and Purcell 2006). The bacterium persists in its vectors during the entire insect life span but is shed with molting (Purcell and Finlay 1979; Purcell et al. 1979; Almeida and Purcell 2006). The loss of vector infectiousness with molting suggests that the foregut is the essential retention site of *X. fastidiosa*. Given the heterogeneous distribution of *X. fastidiosa* in the host plant, and the turbulent rapid flow of xylem-sap into the vector foregut upon uptake, bacterial cells' binding to the foregut is thought to be a rare event, with the majority of the bacterial cells swallowed rather than retained (Retchless et al. 2014). *X. fastidiosa* inoculation positively correlates with the access period to the recipient plant (Hill and Purcell 1995; Almeida and Purcell 2003), the number of infective vectors on the host plant (Daugherty and Almeida 2009), and the number of probes performed by the single infective vector (Jackson et al. 2008). Bacterial inoculation can occur as soon as one hour after acquisition, thus bacterial multiplication and biofilm formation are not required (Purcell and Finlay 1979). Backus et al. (2009) proposed that vectors introduce *X. fastidiosa* into plants through a mechanism defined as "salivation-ingestion-egestion": once stylets reach a xylem vessel, the insect might ingest a mixture of saliva (previously secreted during the formation of the salivary sheath) and xylem sap that is swished through the pre-cibarium and sensed by the pre-cibarial sensilla. This process could lead to an enzymatic (saliva) and mechanical (fluid turbulence) detachment of X. fastidiosa cells within the foregut. These loosened cells could be inoculated into the xylem vessel through egestion, the putative active expulsion of fluid from the food canal (Ramirez et al. 2008; Backus et al. 2012; Backus 2016). Although indirect evidences support this theory, a final correlation between the occurrence of such sequence of behaviors and *X. fastidiosa* inoculation to a recipient plant is missing (Almeida 2016). Identifying the inoculation mechanism of a plant pathogen by its vector involves the real-time observation of the probing behavior of an infective insect given access to a healthy plant; the

83 probe should be terminated when the putative inoculation behavior is performed (Backus
84 2016). The EPG (Electrical Penetration Graph) is a technique that permits the real-time
85 monitoring of hemipterans' probing and feeding behavior (McLean and Kinsey 1964; Tjallingii
86 1978; Backus and Bennett 2009); the use of the EPG has been crucial in determining the
87 behaviors associated with acquisition and inoculation of several vector-borne plant pathogens
88 (Prado and Tjallingii 1994; Martin et al. 1997; Moreno et al. 2012; Antolinez et al. 2017;
89 Jimenez et al. 2018). However, similar studies on *X. fastidiosa* have failed because of the very
90 low inoculation efficiency per individual vector and per probe (Backus 2016). Regarding *the X.*
91 *fastidiosa*-vector relationship, the idea of the insect as a mere carrier of the bacterium has
92 been recently challenged by the finding of bacterial exploitation of vector cuticle as carbon
93 source, with possible detrimental effects for the insect (Killiny et al. 2010; Labroussaa et al.
94 2017). However, to the best of our knowledge, no qualitative or quantitative data on bacterial-
95 mediated effects on the behavior of infective vectors have been produced so far. Such effects
96 of the bacterium on its vectors may have direct consequences on the epidemiology of *X.*
97 *fastidiosa*-related diseases. Most of the background on *X. fastidiosa* transmission dynamics
98 and bacterium-vector interactions exposed above come from studies on the *X. fastidiosa*-
99 grapevine-sharpshooters pathosystem in California (USA) (Rapicavoli et al. 2018). However,
100 vectors other than sharpshooters, i.e. spittlebugs, seem to play the key role in *X. fastidiosa*
101 spread in Europe (Cornara et al. 2018a; Cornara et al. 2019). Indeed, the meadow spittlebug
102 *Philaenus spumarius* L. (1758) (Hemiptera: Aphrophoridae) has been proven to be the main
103 vector of *X. fastidiosa* to olive in South Italy, and is likely involved in bacterial spread in all the
104 European outbreaks reported so far (Saponari et al. 2014; Cornara et al. 2017a; Cornara et al.
105 2017b; Cruaud et al. 2018; Morente et al. 2018; Cornara et al. 2019). *P. spumarius* has some
106 different features with respect to its relationship with *X. fastidiosa* compared to
107 sharpshooters. These differences may relate to spittlebug feeding behavior and the dynamics
108 of fluids within the foregut (Cornara et al. 2016; Cornara et al. 2018b; Sicard et al. 2018;
109 Ranieri et al. 2019). Such differences might have major implications on the spittlebug-
110 mediated transmission of the bacterium that could so far differ in some extent to what has
111 been described for sharpshooters. Therefore, *X. fastidiosa* transmission dynamics by *P.*
112 *spumarius* and spittlebug-bacterium interactions must be investigated in detail. We began to
113 explore the transmission dynamic of *X. fastidiosa* by *P. spumarius* by using EPG in experiments
114 to study the relationship of vector feeding behavior to transmission. We addressed three main
115 questions: i) what is the behavior/sequence of behaviors leading to bacterium acquisition by
116 the meadow spittlebug?; ii) what is the *P. spumarius* behavior/sequence of behaviors leading
117 to bacterium inoculation to the host plant?; iii) are there any differences in probing and
118 feeding behavior between infective and non-infective P. spumarius? The data presented here
119 constitute an essential step for research on spittlebugs transmission of *X. fastidiosa*.

120

121 **Materials and methods**

122 **Collection and rearing of *Philaenus spu*marius**

123 *Philaenus spumarius* individuals used to study the acquisition behavior and for comparison of
124 the feeding behavior of infective versus non-infective spittlebugs were collected at the
125 nymphal stage with a fine-tip brush on ground-vegetation in a *X. fastidiosa*-free olive orchard
126 in Apulia region (Southern Italy) on March 2018. The nymphs were reared until adulthood on a
127 mix of different suitable plant species (*Conyza* sp., alfalfa (*Medicago sativa*), oat (*Avena* sp.)
128 sp., vetch (*Vicia sativa*)) inside a cage (2x1x1m) placed beneath an olive tree inside an
129 experimental field in the premises of the Campus of the University of Bari (Apulia region,
130 Southern Italy). Three to four weeks after emergence, adult spittlebugs (males and females)
131 were collected using a mouth aspirator, transferred to a plastic aerated empty cylinder, and
132 moved to an indoor facility located in the *X. fastidiosa* infected area (Racale (LE), South Italy)
133 where the transmission experiments took place. Before the experiments, the spittlebugs were
134 pre-screened for *X. fastidiosa* by caging them on periwinkle (*Catharanthus roseus*) plants, in
135 groups of five per plant, for an IAP of four to seven days, inside an insect-proof air-conditioned
136 chamber (25±2°C, 40% HR). The pre-screened periwinkles, tested for *X. fastidiosa* ca. 50 days
137 after the IAP by qPCR (following the protocol by Loconsole et al. (2014)), were negative for the
138 bacterium.

139 To assess the inoculation behavior, *P. spumarius* adults (males and females) were collected in
140 the *X. fastidiosa* infected area (Salve (LE), Apulia region). Briefly, in August 2017 insects were
141 collected by sweep net and mouth aspirator in an olive grove with a high disease prevalence
142 (ca. 80% of the olives exhibiting clear symptoms of Olive Quick Decline Syndrome caused by *X.*
143 *fastidiosa*), mainly on the bordering trees and shrubs (oak (*Quercus ilex*), lentisk (*Pistacia*
144 *lentiscus*), persimmon (*Diospyros kaki*), pomegranate (*Punica granatum*), and cypress
145 (*Cupressus sempervirens*)). The spittlebugs were then caged in groups of six per plant on 15-
146 days old vetch plants inside an insect-proof air-conditioned chamber (25±2°C, 40% HR) until
147 the transmission experiments were performed. In 2018, insects were collected in the same
148 olive grove but during the months of June and July, and on olive plants (*Olea europaea*) in
149 addition to the plants described above for the 2017 collection.

150

151 **Experimental plants**

152 Seedlings of *Conyza* sp., alfalfa, oat, and vetch were used to rear the juveniles, while plants of
153 vetch were used to maintain the adult spittlebugs until the EPG experiments. Source plants for
154 the acquisition of the bacterium (*X. fastidiosa* subsp. *pauca*, ST53) consisted of olive seedlings
155 infected in 2014 (transmission experiments details described by Cornara et al. 2017b). The
156 recipient plants used for the inoculation varied according to the EPG experiment and consisted

157 of (i) two-year old olive seedlings (20-30 cm height); (ii) 4-month old self-rooted oleander
158 plants, and (iii) 3-month old periwinkle plants. The plants used for the EPG-assisted
159 transmission experiments were grown in soil, sand and vermiculite (6:3:2).

160

161 ## *Philaenus spumarius* probing and feeding behavior: EPG waveforms

162 The probing and feeding behavior of *P. spumarius* has been characterized through a
163 combination of EPG, video-assisted observations and micro-computed tomography (Cornara
164 et al. 2018b). Five distinct main EPG waveforms were described in that study, with each
165 waveform corresponding to a different behavior during the probe: C (pathway waveform,
166 corresponding to stylets penetration activities during the pathway phase, salivation and build-
167 up of the salivary sheath, and tissue exploration while stylets move toward the xylem vessels);
168 Xc (xylem contact/pre-ingestion, representing the first contact with a xylem vessel and possible
169 pre-ingestion or trial ingestion); Xi (active xylem sap ingestion); R (a resting phase alternated
170 with xylem ingestion); N (brief interruption during the xylem phase, either Xc or Xi, of
171 unknown biological meaning. Waveform N is not considered a “proper” interruption of xylem
172 activities, i.e. in case of occurrence of N, the activities preceding and following N are not
173 calculated as separated xylem contact or ingestion bouts/events (Cornara et al. 2018b)). These
174 patterns are always (or almost always) displayed during all *P. spumarius* probes, as observed
175 on olive, grapevine, vetch and other plants (Cornara et al. 2018b; Markhaiser et al. 2019).
176 Other occasional patterns not previously described represent exceptions to these
177 stereotypically repeated events. Therefore in this work, for the nomenclature of the
178 waveforms (thus the behavioral patterns), we adopted the one used by Cornara et al. (2018b)
179 specifically for *P. spumarius*. Main waveforms produced by *P. spumarius* are reported in Fig. 1.

180

181 ## Acquisition behavior

182 After pre-screening, non-infective adult spittlebugs were transferred to two weeks old vetch
183 plants (non-host of the ST53 strain used in this experiment), in groups of ten per plant, inside
184 an insect-proof air conditioned chamber (same conditions described for pre-screening) until
185 the EPG-assisted AAP (Acquisition Access Period) (one to 14 days). The probing and feeding
186 behavior of pre-screened insects on *X. fastidiosa* olive source plants was monitored through
187 EPG, in order to identify the behavior(s) associated with bacterial acquisition. There were four
188 treatments, with at least 30 replicates per treatment: interruption of the probe during
189 pathway (C); interruption of the probe during xylem contact (Xc); one hour AAP on an olive
190 infected plant; three hours of AAP on an olive infected plant (Tab. 1). *X. fastidiosa* source
191 plants for the EPG-assisted AAP were three infected olives showing approximately the same
192 vegetative conditions. The plants were trimmed at 30 cm of height one week prior to the

193 beginning of the experiment, and pruned leaving non symptomatic green lateral shoots more
194 suitable for meadow spittlebug settling and probing (Cornara et al. 2018a). For each of the
195 infected-source plants, we selected a middle non-symptomatic shoot; one week before the
196 EPG, half of the leaves of the selected shoots were tested by qPCR (following the protocol by
197 Loconsole et al. (2014)) and found positive to the bacterium. Additionally, at the end of the
198 acquisition experiment, the shoots offered to the spittlebugs were re-tested by qPCR (pooling
199 together the leaves and the stems); all the tissues selected for the acquisition tests showed
200 similar bacterial population (ranging from 8.34E+04 to 3.38E+05 CFU/ml). We EPG-recorded
201 three spittlebugs per time, each on one EPG-channel and on one source plant, and all the
202 three subjected to the same treatment (interruption of the probe during the waveform C, or
203 Xc, or after 1h or 3h). Following the EPG-assisted AAP, each spittlebug was gently removed
204 from the infected-source plant with a paint brush, and caged on a non-infected periwinkle
205 plant for an IAP of 96 hours, inside an insect proof air-conditioned chamber (25±2°C, 40% HR).
206 At the end of the IAP, the insects were collected and stored in ETOH 70% at -20°C; the
207 receptor plants were maintained in an insect proof air-conditioned chamber at 27±2°C 40%
208 HR, and watered twice a week. Insect infectivity and periwinkle infection status (the latter
209 assessed ca. 40 to 60 days after the IAP) were tested by qPCR, following the protocols by
210 Harper et al. (2010) and Loconsole et al. (2014). We considered acquisition as having occurred
211 if at least one of the two samples per replicate (the insect and the receptor plant) tested
212 positive by qPCR.

213

214 **Inoculation behavior**

215 The spittlebugs were given a seven to ten-day AAP on five infected olive seedlings inside a
216 Bugdorm-2 Insect tent (https://shop.bugdorm.com). Following the AAP on the infected olive
217 plants, the spittlebugs were moved to healthy vetch plants until the EPG-assisted-inoculation
218 tests began (the spittlebugs remained caged on the healthy vetch plants approximately from
219 two to 20 days after the AAP on infected olive plants). After tethering and connection to the
220 EPG probe, each *P. spumarius* was placed on a 5 cm portion of a healthy olive seedling stem,
221 having access to at least one leaf. The probing and feeding behavior of *P. spumarius* on olive
222 receptor plants was monitored through EPG, in order to identify the behavior(s) (EPG's
223 waveform(s)) associated with bacterial inoculation. Each spittlebug was left probing until the
224 occurrence of the waveform of interest; once the waveform occurred, the insect was removed
225 from the receptor plant with a paint brush, and stored in ETOH 70% at -20°C until the
226 assessment of its infectivity. In the 2017 EPG experiments, there were four treatments with
227 termination of probing during different EPG waveforms: pathway (C); xylem contact (Xc);
228 xylem ingestion (Xi; from five to 15 minutes); first interruption during xylem activity (N) (either
229 during Xc or Xi) (Tab.2A). After each replicate, the probed olive portion (ca. 1cm) was marked

230 with tape; the plants were stored in an insect proof air-conditioned chamber at 27±2°C 40%
231 HR, and watered once a week. The recipient plants were tested three months after the EPG-
232 assisted IAP; inoculation of bacterial cells into the xylem was assessed by qPCR on either the
233 probed part of the seedling (2cm portion, both stem and at least one leaf petiole), or a portion
234 three to four cm distal to the former (2cm portion, both stem and at least one leaf petiole).
235 Moreover, in 2017 we had an additional treatment: some of the insects were given a one-hour
236 EPG-assisted IAP (insects tethered and connected to the EPG) on the receptor test plant
237 without artificially interrupting the probe during a certain waveform (Tab. 2A). The plants
238 were maintained at the same conditions described above for the recipient plants used for
239 waveform interruption IAP; plant status was ascertained by qPCR three months after the IAP,
240 testing a portion of ca. 10 cm including the five cm stem/leaves exposed to *P. spumarius*
241 probing. The sample was not split in two (probed and distal parts) as for the waveform-
242 interruption treatments, since the spittlebugs were allowed to make multiple probes. For the
243 2017 experiment, we performed 25 replicates for each waveform interruption treatment, and
244 30 for the 1 h IAP, which included both infective and non-infective *P. spumarius*. Tab.2A
245 reports results only from replicates where spittlebugs were positive for *X. fastidiosa* by qPCR
246 (with a Ct approximately ranging from 26 to 32). None of the recipient plants that were
247 exposed to spittlebugs found to be non-infective according to qPCR results tested positive for
248 *X. fastidiosa*.

249 In 2018, treatments in the inoculation tests were either interrupting the probe during the
250 waveform of interest, or giving the spittlebug an IAP of three hours on the olive recipient
251 plant. The waveform interruption treatments were: pathway (C); xylem contact (Xc); xylem
252 ingestion (Xi; from five to 15 minutes), either with or without xylem activity interruptions (N);
253 interruption during xylem activity (N); resting (R; from one to two minutes) (Tab.2B). For the
254 2018 inoculation experiment, there were 30 to 60 replicates for each of the waveform-
255 interrupted treatments, and 90 replicates for the 3 h IAP. In Tab.2B we reported only the
256 plants exposed to infective *P. spumarius* according to qPCR (with Ct values approximately
257 ranging from 24 to 33). None of the plants exposed to non-infective spittlebugs (as determined
258 by qPCR) tested positive for *X. fastidiosa*. In addition to olive, in 2018 we performed also EPG-
259 assisted waveform interruption inoculation tests on 4-month old oleander plants. As shown by
260 Cornara et al. (2017b), *X. fastidiosa* inoculation rate to oleander by *P. spumarius* is greater
261 than to olive, despite oleander being a very poor host for the spittlebug. Furthermore, *P.*
262 *spumarius* on oleander performs single or repeated unconventional and "occasional" EPG
263 signals different from the stereotypically repeated patterns (C, Xc, Xi, R, N) far more frequently
264 than in olive and other plants (vetch, grapevine, cherry) (Cornara et al. 2018b; Markheiser et
265 al. 2019). These unconventional EPG patterns, occurring from seconds to few minutes after
266 the insect has reached the xylem vessel, include a spikelet burst similar to the B1s waveform
267 described for sharpshooters (Backus et al. 2009; Backus et al. 2005; Joost et al. 2006) (Fig.1f

268 and Fig.2a and b), and a voltage drop similar to N but occurring during an initial resting phase
269 alternated with low frequency Xi (frequency≤0.1Hz) (Fig.2c). Here we grouped these two EPG
270 patterns under a single treatment, provisionally termed Xe. Therefore, for the waveform
271 interruption inoculation tests on oleander, we added the treatment Xe to those described for
272 olive (Tab.2C); Xe was not produced by spittlebugs on olive during the waveform interruption
273 experiments carried out either during 2017 or 2018.

274 For the 2018 EPG-assisted inoculation tests, and insects and plant maintenance, we followed
275 the same protocol described above for the 2017 experiments. The waveform-interruption
276 recipient plants (both olive and oleander) were tested three months after the EPG-assisted
277 inoculation tests; the presence of bacterial cells in the recipient plants was assessed by qPCR
278 on either the probed part, or a portion three to four cm distal to the probed part. For the olive
279 recipient plants where *P. spumarius* had an EPG-assisted 3-h IAP on, we tested a portion of ca.
280 10 cm including the 5 cm stem/leaves exposed to *P. spumarius* probing; as for the 1-h IAP of
281 2017, we did not split the sample in two.

282 For qPCR on insects and plants, we followed the protocols described by Harper et al. (2010)
283 and Loconsole et al. (2014), respectively.

284 As explained above, during both 2017 and 2018, we added to the waveform-interruption
285 experiments further treatments, namely 1h IAP (2017) and 3h IAP (2018), without interruption
286 of the probe during specific waveforms. We decided to use relatively short IAPs for two main
287 reasons: i) as remarked by Wayadande and Nault (1993), long feeding periods result in more
288 switching from one behavior to another, making it difficult to know which behavior(s) is/are
289 associated with pathogen inoculation; ii) several indirect evidences suggest that *X. fastidiosa*
290 inoculation might occur during the initial steps of the probe (Jackson et al. 2008; Daugherty
291 and Almeida 2009; Backus et al. 2009; Backus 2016).

292

293 **Comparison of infective versus non-infective *Philaenus spumarius* probing behavior**

294 To compare the feeding behavior of infective *versus* non-infective *Philaenus spumarius*, adult
295 females were used. The insects were given a 10-day AAP on five infected olive seedlings inside
296 a Bugdorm-2 Insect tent (https://shop.bugdorm.com). Following the AAP, the spittlebugs were
297 moved to healthy vetch plants until the EPG-assisted IAP (from one to 7 days). After tethering
298 and connection to the EPG, each spittlebug was given a 3h IAP on a 5 cm portion of a stem of a
299 healthy olive seedling, having access to at least one leaf. We selected a 3-h IAP in order to be
300 consistent with the protocol used for the 2018 inoculation behavior experiment. Following the
301 EPG recording, each spittlebug was caged on a healthy periwinkle plant for an IAP of 96 hours,
302 inside an insect proof air-conditioned chamber (T=25±2°C, HR=40%). At the end of the IAP, the
303 insects were collected and stored in ETOH 70% at -20°C; the plants (olives and periwinkles)

304 were maintained in an insect proof air-conditioned chamber at 27±2°C 40% HR, and watered
305 twice a week for periwinkles, once a week for olives. Insect infectivity and plant infection
306 status (the latter assessed ca. 50 days after the IAP for periwinkles, and three months for
307 olives) were tested by qPCR, according to Harper et al. (2010) and Loconsole et al. (2014),
308 respectively. For olive, we tested a portion of ca. 10 cm including the 5cm stem/leaves part
309 exposed to *P. spumarius* probing. Each insect given the 10-days AAP on olive infected plants
310 was considered infective if at least one of the two samples per each replicate (either the insect
311 or the periwinkle recipient plant) tested positive for *X. fastidiosa* by qPCR.

312

313 **EPG procedure and data analysis**

314 For running the EPG tests, the insects were: 1) starved for one hour inside an aerated Petri
315 dish; 2) slightly stunned by exposure to 4°C for ca. 30 sec.; 3) immobilized with a cased
316 diaphragm pump (Dymax 5, Charles Austen Pumps Ltd, Byfleet, Surrey, England/UK); 4)
317 tethered according to the protocol described by Cornara et al. (2018b). Briefly, the tip of an 18
318 µm-gold wire, 3 cm long, was placed on the insect pronotum, and glued with a double layer of
319 silver conductive glue (Ted Pella, no. 16034; Pelco® Colloidal Silver, Ted Pella, Redding, CA,
320 USA). The tip of the wire was bent in order to create a loop that enhanced the resistance of
321 the connection. The other end of the wire had been attached previously with silver paint to a
322 copper electrode measuring 3 cm in length × 1 mm in diameter. Thereafter, the electrode was
323 plugged into the EPG probe, with the insect left hanging over the plant without touching it for
324 ca. ten minutes before placing it on the plant. The soil copper electrode (10 cm long × 2 mm
325 wide) of the EPG device was then inserted into the pot substrate. The system was assembled
326 inside a Faraday cage, in an acclimatized room (25 ±2°C), and under artificial light (20W, 1200
327 Lm (lumen)). Probing and feeding behavior was recorded with a Giga 4-DC EPG device (EPG-
328 systems, Wageningen, The Netherlands) with 1 Giga Ohm input resistance. Output from the
329 EPG at 100x gain was digitalized at a rate of 100 samples per sec. per channel, and recorded
330 using Stylet+ software (EPG-systems, Wageningen, The Netherlands). Substrate voltage was
331 adjusted following the calibration instructions of the DC EPG equipment so that EPG signals fit
332 into the +5V to -5V window provided by the software Stylet+ (EPG-systems, Wageningen, The
333 Netherlands). The EPG recordings were analyzed by Stylet+. For the acquisition experiment
334 (Tab.1) and the comparison between infective and non infective *P. spumarius* (Tab.3), several
335 sequential and non-sequential variables were calculated. The variables calculated and the
336 abbreviations used in tables 1, 2, and 3 are described in Tab.4. EPG variables were calculated
337 with an Excel Workbook developed purposely for *P. spumarius* waveforms by Antonio J.
338 Alvarez (Universidad de Almeria, Spain) (Cornara et al. 2018b). Differences in probing and
339 feeding behavior between infective and non-infective spittlebugs were assessed by Mann-

340 Whitney U-test. Statistical analysis was performed with the software R 3.5.2 (R Core Team,
341 2015).

342 **Results**

343 **Feeding behavior associated with the acquisition/retention of *X. fastidiosa* by *P. spumarius***

344 Two *P. spumarius* individuals only, one given 1h AAP and the other given a 3h AAP tested
345 positive for *X. fastidiosa* by qPCR (Ct=31.67 and 31.34) (Tab. 1); no transmission to periwinkle
346 occurred. None of the spittlebugs whose probe was interrupted during pathway (C) or xylem
347 contact (Xc) phases acquired and retained the bacterium. The extremely low acquisition rate
348 did not permit any statistical inference; nevertheless, we analyzed the sequence of events and
349 calculated non-sequential variables for the two replicates that acquired the bacterium, in
350 order to have preliminary indications about the feeding activities associated with the
351 acquisition and retention of *X. fastidiosa* (Tab.1). Considering the acquisition that occurred in
352 the 1h treatment, the spittlebug performed a single probe of 31.2 min, of which 14.8 min
353 spent in xylem ingestion and xylem interruption activities (2.3 of the 14.8 min of Xi were spent
354 in N; 12 N waveforms were performed), and 1.2 min in resting. The spittlebugs that acquired
355 the bacterium in the 3h treatment performed a long xylem ingestion phase (122.8 min), with a
356 single xylem interruption, and a resting phase that lasted 0.8 min.

357

358 **Feeding behavior associated with the inoculation of *X. fastidiosa* by *P. spumarius***

359 Considering the waveform-interruption experiments, no inoculation to olive was obtained in
360 2017 and in 2018 by interrupting the probes during the occurrence of the patterns C, Xc, Xi
361 (whether or not containing from one to three interruptions N), N or R (Tab.2A and 2B). On
362 oleander, five *P. spumarius* positive for *X. fastidiosa* to qPCR produced the pattern Xe (namely
363 one of these five spittlebugs performed a voltage drop, and 4 spittlebugs performed each a
364 spikelet bursts). Three of these five spittlebugs, i.e. one performing a drop and two producing
365 spikelet bursts, inoculated *X. fastidiosa* to the receptor plant (Tab.2C). Both the probed and
366 the distal parts of each of the inoculated oleanders were positive for the bacterium by qPCR,
367 indicating that bacterial cells were released into the xylem. The voltage drop performed by the
368 spittlebug that successfully inoculated the recipient plant occurred 7 minutes after the
369 beginning of the probe, and 3 minutes after the first contact with xylem. The two spikelet
370 bursts in the spittlebugs that inoculated the plants occurred two and two and a half minutes
371 after the beginning of the probe, and 0.5 and 1 minutes after the xylem contact. No
372 inoculation to oleander occurred with the other patterns tested (Tab.2C). Considering the 1-
373 hour IAP on olive, the two spittlebugs (out of the 12 infective insects) that were able to infect
374 the plants were the only ones that produced an Xe pattern (one voltage drop occurring 6
375 minutes after the onset of the probe, and 5 minutes after the contact with xylem; one spikelet
376 burst performed 2.5 minutes after the beginning of the probe, and 2 minutes after the xylem
377 contact). Finally, considering the 3-hours IAP, one inoculation out 49 infective *P. spumarius*

378 was obtained. The inoculative spittlebug was one of the only three insects (out of the 49
379 infective) producing Xe (a drop in R occurring 3 minutes after the onset of the probe, 1 minute
380 after the first contact with xylem); the other two, producing spikelet bursts, did not transmit *X.*
381 *fastidiosa* to the host plant.

382

383 **Comparison of infective versus non-infective *Philaenus spumarius* probing behavior**

384 We included in the analysis only clear recordings (without noise or unclear signals) performed
385 by *P. spumarius* that: i) remained on the plant for the 3h of EPG without breaking the wire and
386 escaping or falling off of the host; ii) were alive and active at the end of the IAP on periwinkle;
387 iii) probed the tissue at least once during the recording. By these criteria, 49 *P. spumarius*
388 females, 14 infective and 35 non-infective were selected for statistical analysis. Nine out of 14
389 *P. spumarius* positive to the bacterium by qPCR transmitted *X. fastidiosa* to the periwinkle
390 recipient plants. Two out of the 14 infective spittlebugs inoculated the fastidious bacterium
391 during the EPG-assisted three hours IAP to olive; the limited number of inoculations did not
392 permit any statistical inference. Looking at the behavioral patterns displayed during the
393 probes, the waveform Xe was performed only by the two insects that inoculated *X. fastidiosa*
394 to olive (one spittlebug producing a drop and one a spikelet burst both occurring ca. 4 minutes
395 after the beginning of the probe, and 3 minutes after the xylem contact). A third spittlebug
396 performing a spikelet burst did not inoculate the bacterium. Sequential and non-sequential
397 variables calculated for the infective and non-infective spittlebugs are reported in Tab.3.
398 Infective *P. spumarius* spent significantly longer time in non-probing (W=340, p=0.036) and
399 shorter time in xylem ingestion (W=136, p=0.016) activities compared to non-infective
400 spittlebugs. Furthermore, we observed also that the average duration of the single non-
401 probing events in infective insects was almost twice the value recorded for non-infective ones
402 (W=350, p=0.020). Moreover, infective *P. spumarius* performed significantly fewer sustained
403 xylem ingestion events, i.e. xylem ingestions longer than 10 minutes (W=152.5, p=0.032) and
404 interruptions of the xylem activity (waveform N) (W=146, p=0.027) than non-infective. Finally,
405 infective spittlebugs required longer time to perform the first probe compared to individuals
406 not carrying the bacterium (W=346.5, p=0.024).

407

408 **Discussion**

409 The data presented here can guide further attempts to determine the vector feeding
410 behaviors necessary for *P. spumarius* transmission of *X. fastidiosa* to plants. Our principal
411 conclusions were that: i) spittlebug acquisition rate appeared to be extremely low, and
412 bacterial cells binding to the foregut might occur in a time as short as 15 minutes spent by the
413 insect performing xylem ingestion, or other activities interspersed with xylem ingestion
414 (interruption or resting); ii) inoculation of bacterial cells into the host plant xylem by *P.*
415 *spumarius* was associated with an early and very occasional waveform that we provisionally
416 termed Xe (that occurred ca. 2 to 7 minutes after the onset of the probe). The common
417 feeding behavioral patterns, i.e. C, Xc, Xi, N, R, that the spittlebugs stereotypically repeat
418 during most of the probes, were not associated with bacterial cells delivery to the host plant.
419 Our hypothesis is that Xe waveform likely represents egestion of fluids regulated by the pre-
420 cibarial valve fluttering following a possible lack of insect phagostimulation. However, the low
421 inoculation rate displayed by *P. spumarius* during our experiments make it difficult to draw a
422 definitive conclusion about the exact behavior associated with bacterial cells inoculation, and
423 more research efforts are needed; iii) probing and feeding behavior of infective *P. spumarius*
424 differed from the one of non-infective spittlebugs. The EPG analysis showed that infective *P.*
425 *spumarius* had more difficulties than non-infective ones in feeding on a non-infected host
426 plant.

427 **Feeding behavior associated with acquisition/retention of X. fastidiosa**

428 The interaction between two main factors makes *X. fastidiosa* acquisition and retention within
429 the vector foregut a relatively rare event: first, the bacterium is unevenly distributed within
430 the plant, thus for acquisition (uptake) to occur the insect should probe from one of the
431 vessels colonized by the bacterium (Hopkins 1981; Newman et al. 2003; Cardinale et al. 2018);
432 second, the xylem sap flows within the insect foregut at an extremely high velocity, generating
433 turbulence, thus hindering the bacterial cells attachment (Purcell et al. 1979; Dugravot et al.
434 2008). Therefore, even if the insect lands on an infected plant, and probes a vessel containing
435 *X. fastidiosa* cells, most of the cells up-taken would be swallowed without being retained in
436 the precibarium (Retchless et al. 2014). However, it is expected that long access periods could
437 increase the probability of vector-pathogen encounters, overall increasing the acquisition rate
438 (Almeida 2016). Our data suggest that *X. fastidiosa* acquisition and retention by *P. spumarius*
439 do not necessarily require very long probe, and likely occur during xyle ingestion (waveform Xi)
440 from infected vessels; a xylem ingestion as short as 15 minutes is sufficient for successful
441 binding. Therefore acquisition and successful retention might occur during the xylem
442 ingestion, with cells binding during the simultaneous collapse of the cibarial diaphragm and
443 closure of the precibarial valve sealed by the bell-like invagination (Ruschioni et al. 2019), or
444 during activities interspersed with xylem ingestion, namely xylem interruption N (a single

445 interruption could be sufficient) or resting. The extremely low acquisition rate we observed for
446 the meadow spittlebug is consistent also with previous data by Cornara et al. (2016). However,
447 our experiments did not permit any statistical inference, or to draw conclusions about the
448 precise behavior(s) or sequence of events leading to *X. fastidiosa* acquisition. Furthermore, the
449 low acquisition rate could have been influenced by the relatively low bacterial population
450 within our olive source plants, given the positive correlation between *X. fastidiosa* population
451 within the infected plant and the transmission efficiency ((Hill and Purcell 1997). Nevertheless,
452 high *X. fastidiosa* population lead to symptoms development, and the vectors tend to
453 discriminate against symptomatic plants (Marucci et al. 2005; Miranda et al. 2013; Zeilinger
454 and Daugherty 2014; Del Cid et al. 2018). Therefore, considering our scenario, consisting of
455 infected but non-symptomatic plants bearing a bacterium population still too low to cause
456 severe symptoms and consequent reduction of host plant attractiveness, the most
457 epidemiologically realistic for inferences on acquisition dynamic. However, further
458 experiments either with olive or with other host plants should be conducted to deepen our
459 knowledge about the mechanism of acquisition of *X. fastidiosa* by *P. spumarius*, and about
460 how and where bacterial cells do initially bind to the spittlebug foregut.

461

462 **Feeding behavior associated with the inoculation of *X. fastidiosa***

463 *X. fastidiosa* cells delivery into the xylem vessels by *P. spumarius* was associated with the
464 occurrence of a pattern that we provisionally called Xe, as demonstrated by: i) the successful
465 inoculation to oleander plants only when the probe was interrupted in correspondence of this
466 particular pattern; ii) the only inoculations to olive occurred during IAPs where the spittlebugs
467 engaged in Xe; iii) the lack of inoculation with the other behavioral patterns tested either on
468 olive or on oleander. In other words, whenever there was infection of test plants, spittlebugs
469 always made at some point an Xe waveform on receptor test plants. The spittlebug performed
470 this specific behavior 2 to 7 minutes after the beginning of the probe, and 0.5 to 5 minutes
471 after the first contact with the xylem. Furthermore, in all the observed cases in olive, the
472 pattern Xe was always preceded by waveforms C (pathway), Xc (xylem contact) and Xi (xylem
473 ingestion activity); in oleander, Xe was preceded by the sequence of events C-Xc in two out of
474 three inoculative probes, and by C-Xc-Xi in the other positive case. The pattern Xe was never
475 preceded by the xylem interruption N waveform. No inoculation occurred when infective
476 spittlebugs probe was interrupted during waveforms C, Xc, or Xi, neither in olive nor in
477 oleander. Under the term Xe we grouped two apparently different EPG signals, a voltage drop
478 occurring during a period where resting (R) alternates with low frequency Xi (frequency≤0.1
479 Hz) (Figs.2c and f), and a “simple” spikelet burst (Fig.1 f and Figs.2a, b, d, e). The common
480 element between the two signals is the presence of spikelet bursts (indicated with arrows in
481 Figs. 2a, b and c), characterized by highly variable frequency (3 to 10 Hz) and amplitude (4 to

482 25%). During voltage drops, spikelet bursts were repetitive and no longer than 1-2 seconds,
483 while the duration of the "simple" spikelet bursts ranged between 6 and 17 sec. This similarity
484 suggests that the inoculation of *X. fastidiosa* cells into the plant by *P. spumarius* could be
485 associated with the spikelet bursts occurring when the insect stylets are located in a xylem
486 vessel, after having built the salivary sheath, penetrated through the plant tissues reaching a
487 xylem vessel, and after a first tasting of the host plant suitability through the pre-cibarial
488 chemosensilla. According to Joost et al. (2006) and Backus et al. (2009), spikelet burst (termed
489 B1s in these and in further works on sharpshooters performed by Backus and colleagues)
490 represents an insect internal activity, possibly streaming potentials (Walker 2000) caused by
491 pre-cibarial valve movements defined as fluttering. This behavior is interspersed during the
492 probe, and occurs frequently during the pathway phase before reaching the xylem vessel; its
493 occurrence may therefore be associated with movements of the pre-cibarial valve during
494 tasting of the host plant (Backus et al. 2009; Backus and McLean 1982; Backus 1985). For a
495 thoroughly review of the different waveforms subtypes in sharpshooters refer to Backus
496 (Backus 2016). As shown in our experiment, at least for *P. spumarius*, the occurrence of
497 spikelet bursts when the insect stylets are located in a xylem vessel, thus putative pre-cibarial
498 valve fluttering within the xylem vessel pushing bacterial cells out of the food canal possibly
499 helped by the tension of the xylem fluid while the insect is feeding, may lead to *X. fastidiosa*
500 inoculation. Pre-cibarial valve involvement in *X. fastidiosa* inoculation has also been proposed
501 by other authors (Purcell et al. 1979; Almeida and Purcell 2006). Spikelet bursts are also major
502 components of the X-waveform found to be associated with the inoculation of the Maize
503 Chlorotic Dwarf Virus (MCDV, Waikavirus), a semi-persistent virus sharing with *X. fastidiosa*
504 the characteristic of being foregut-borne ((Childress and Harris 1989; Ammar and Nault 1991;
505 Wayadande and Nault 1993). Wayadande and Nault (1993) suggested that the biological
506 meaning of the X-waveform is egestion (*sensu* Harris (1977); termed extravasation by McLean
507 and Kinsey (1984)), the delivery of plant fluids present within the food canal anterior to the
508 cibarial pump back to the stylets and then into the plant, occurring when the plant fluid itself
509 fails to induce phagostimulation (McLean and Kinsey 1984).

510 Theoretically, valve fluttering occurring during the voltage drop inside a resting/low frequency
511 Xi phase (when stylets are inside the xylem), even if shorter than "simple" fluttering (not
512 occurring during a drop), would generate a force sufficient to egest bacterial cells from the
513 foregut to the plant. Indeed, during the resting phase, insect cibarial (and pre-cibarial) muscles
514 are either not contracting, or contracting at a very low frequency (<0.1Hz) (Cornara et al.
515 2018b). Theoretically, the slower a muscle contracts, the greater the internal tension, thus the
516 greater the force it can generate (Malone et al. 1999; Sutton and Burrows 2018). Therefore, if
517 the fluttering occurs after resting, the force generated (likely by the pre-cibarial valve) would
518 be sufficient to propel bacterial cells toward the xylem vessels even if the behavior is
519 performed for a short period. Therefore, the Xe waveform may represent the opening and

520 likely fluttering of the pre-cibarial valve, that propels the bacterial cells toward the xylem
521 vessel possibly helped by the negative tension of the xylem sap.

522 Considering the spittlebugs that had 3h of IAP without interruption of the probe (both the 49
523 infective spittlebugs in the inoculation experiment, and the 14 individuals in the behavioral
524 comparison; Tab.2B), this behavior (Xe) was performed by six out of the 63 infective
525 individuals, leading to successful inoculation in three cases. Therefore, Xe represents a
526 relatively occasional/relatively rare behavior (on olive), given that in our experiment, ca. 9.52%
527 (six out of 63, data not shown in the table) of the infective individuals performed it, and only a
528 half of these individuals (three out of 63, ca. 4.76% of the infective spittlebugs) inoculated the
529 bacterium. This inoculation rate is consistent with data on *P. spumarius* bacterium inoculation
530 to grape, with one out of 30 plants infected by single insects given an IAP of either 1.5 or 4.5
531 hours (Cornara et al. 2016). The association of *X. fastidiosa* inoculation by the meadow
532 spittlebug with a relatively infrequent/occasional behavior is also consistent with the
533 occasional transmissions to grapevine during spittlebugs sequential daily transfer to healthy
534 recipient plants reported by Severin (1950) (infection rate ranging from 5 to 16%). In fact,
535 transmission rate of *X. fastidiosa* by *P. spumarius* is much more inefficient than the rate of
536 transmission of other foregut-borne plant-pathogens such as Beet yellows virus (which is close
537 to 50% by a single aphid) (Jimenez et al. 2018). Therefore, data presented here, supported by
538 the observations by other authors described above, suggest that *P. spumarius* likely inoculates
539 *X. fastidiosa* during the pattern Xe occurring just few minutes after the beginning of the probe,
540 and that, at least on suitable plants as olive, this behavior is a relatively rare event different
541 from the patterns stereotypically repeated by the insect during most of the probes (namely C,
542 Xc, Xi, N, R). Overall, considering not only the Xe behavior, but also the sequence of events
543 preceding it, we propose that the behavior leading to *X. fastidiosa* inoculation into the host
544 plant by *P. spumarius* is egestion driven by pre-cibarial valve fluttering resulting from a failure
545 of insect phagostimulation following the tasting of the host-plant xylem sap, possibly helped
546 by xylem fluid tension while the insect is feeding. Moreover, as discussed in the materials and
547 methods section, such unusual behavior occurs more frequently in oleander than in olive
548 (observed by Cornara et al. 2018b and Markheiser et al. 2019). Cornara et al. (2017b) reported
549 that *P. spumarius* transmission rate to oleander is far greater than to olive, although all the
550 insects on the former host died within 24 hours from caging. Therefore, transmission is
551 apparently enhanced if the spittlebug is forced to feed on an unsuitable substrate, possibly
552 because lack of phagostimulation (or feeding deterrence) and subsequent egestion would be
553 more likely to occur on a less -or not- acceptable host. This hypothesis is also supported by
554 increased rate of transmission by *Homalodisca virtipennis* Germar (1821) (Hemiptera:
555 Cicadellidae) caged on grapevines treated with the insecticide pymetrozine (Bextine et al.
556 2004). Therefore, the behavior associated to *X. fastidiosa* inoculation could be triggered by
557 conditions of the host plant unfavorable for the insect; the identifications of such factors,

558 whether related to the host plant, to the vector, or to the interactions between the two
559 elements, deserve further investigation. Furthermore, the frequency of Xe may also increase
560 because of the presence of the bacterium in the foregut, but this needs further investigation.

561

562 Comparison of infective versus non-infective *Philaenus spumarius* probing and feeding 563 behavior

564 Plant pathogens influence the transmission process, i.e. the recruitment of the vector on the
565 infected plants for acquisition and the successive dispersal for inoculation, via effects on plant
566 or vector phenotypes that modify the nature and the frequency of the interactions between
567 them (Mauck 2016; Mauck et al. 2018). To be categorized as parasite manipulation a
568 documented effect of a plant pathogen on its vector should: 1) enhance, or create conditions
569 expected to enhance transmission; 2) be under genetic control of the pathogen (Mauck et al.
570 2019). Vector transmission may be enhanced by the pathogen through indirect effects, i.e.
571 effects on host derived sensory cues (Eigenbrode et al. 2002; Jimenez-Martinez et al. 2004;
572 Mauck et al. 2010; Shapiro et al. 2012), or direct effects on insects behaviors such as probing
573 and host-searching/dispersal (Stafford et al. 2011; Ingwell et al. 2012; Moreno-Delafuente et
574 al. 2013; Martini et al. 2015). The same effects can be induced by highly divergent pathogens
575 sharing the same mechanism of transmission (Mauck 2016; Stafford et al. 2011; Lefevre and
576 Thomas 2008). According to Moreno-Delafuente et al. (2013), persistent circulative viruses are
577 more likely to influence vector behavior given that the vector-pathogen relationship lasts for
578 the entire insect life span, although semi-persistent viruses effects on vector behavior have
579 been documented (Lu et al. 2017; Pereira et al. 2019). Mauck et al. (2019) suggest proteins
580 encoded by pathogens to facilitate interaction with their vectors following acquisition may be
581 co-opted to induce behavioral changes that enhance transmission. *X. fastidiosa* fulfill both the
582 previously mentioned “requirements”, being persistent in its vectors (Severin 1950; Purcell
583 and Finlay 1979), and encoding proteins necessary for interacting with the insect vector (Killiny
584 and Almeida 2014). Additionally, the fact that *X. fastidiosa* exploits the cuticle of its vectors as
585 a substrate for multiplication, suggests a parasitic relationship, with a negative impact of the
586 bacterium on the insect (Labroussaa et al. 2017). As observed in our experiment, the probing
587 and feeding behavior of infective *P. spumarius* females significantly differs from that of non-
588 infective ones. The main affected behaviors were non-probing and xylem ingestion, with an
589 overall longer time spent in non-probing and a shorter time spent in xylem ingestion by
590 infective insects. Particularly, *P. spumarius* carrying *X. fastidiosa* showed evident difficulties in
591 performing sustained xylem ingestion (ingestion longer than ten minutes), with fewer events
592 compared to healthy insects. Furthermore, infective spittlebugs showed a duration of
593 individual non-probing events twice that of non-infective insects, fewer xylem interruptions N,
594 and longer time before probing the host plant for the first time compared to insects not

carrying the bacterium. Taken together, these observations suggest difficulties in feeding caused by the presence of *X. fastidiosa* within the foregut, similarly to what has been recently hypothesized by Ranieri et al. (2019), possibly caused by a mechanical obstruction of the food canal. However, a biological effect caused directly by *X. fastidiosa* on the insect aimed at creating a favorable environment for the bacterium within its vector cannot be ruled out. Indeed, longer non-probing alternated with short xylem ingestion, thus longer period with almost no muscle contraction, sap flow or turbulence, would represent a perfect condition for bacterial cells to bind, multiply, and colonize the foregut. Such manipulation could either affect vector fitness, or be conducive for transmission. Indeed, as observed for example in mosquitos bearing the malaria Plasmodium, the vector could respond to difficulties in feeding by increasing the number of probes (Lefevre and Thomas 2008). Since, as observed in this study, inoculation of bacterial cells by the meadow spittlebug can occur just a few minutes after the beginning of the probe and is possibly associated with an occasional event (Xe), an increased number of probes could theoretically increase the probability of the inoculation behavior to occur, thus the overall inoculation rate.

Conclusions and further perspectives.

Recent researches on vector-pathogen relationship disruption (Killiny et al. 2012; Labroussaa et al. 2016), bacterium biological control (Baccari et al. 2018), and sources of resistance (Giampetruzzi et al. 2016) offer promising perspectives for a sustainable and effective *X. fastidiosa*-diseases control. However, with regards to the European outbreaks of the bacterium, these perspectives are limited by our lack of knowledge about several pivotal aspects of the epidemics, especially concerning the spittlebugs-bacterium interaction and the spittlebugs-mediated transmission mechanism. Here we began to shed some light on *X. fastidiosa* transmission dynamics by *P. spumarius*, opening at the same time new challenging questions. For example, the identification of conditions triggering the putative egestion behavior associated with bacterial cells inoculation would have interesting implications on sustainable control strategies. The *X. fastidiosa* inoculation behavior should also be characterized on other vector-host plant-strain combinations. Furthermore, an in-deep characterization and description of the waveform Xe and its sub-patterns is absolutely needed.

Considering the relatively low acquisition and inoculation rates displayed by *P. spumarius*, an effective control of the meadow spittlebug populations could result in a significant reduction of the risk of *X. fastidiosa* spread. Indeed, according to Irwin and Ruesink (1986), vector intensity depends on vector propensity (innate ability of the vector to transmit a certain pathogen) and vector activity (number of insect vectors alighting on the host plant for a

631 certain period of time); therefore, a reduction of vector activity would lead to a decrease of
632 vector intensity. However, several aspects related to vector ecology should be investigated in
633 order to develop a sustainable long-term *X. fastidiosa* management strategy: i) vector
634 population abundance within the orchard; ii) factors driving vector host selection and within-
635 host plant preference; iii) vector aggregation and dispersal dynamics; iv) influence of
636 landscape on vector population dynamics (Santoiemma et al. 2018; Bodino et al. 2019).

637 Finally, other challenging questions come from our finding about differences in probing and
638 feeding behavior between infective and non-infective *P. spumarius*; we discussed above how
639 these differences could be beneficial to the bacterium and detrimental for the spittlebug.
640 However, we recognize the limits of our experimental approach (we used only females,
641 monitored for a relatively short period (three hours) and with the bacterium acquired from the
642 infected plant). This does not permit drawing conclusions about pathogen manipulation
643 exerted by the bacterium on the spittlebug. More research efforts should be put in place to
644 thoroughly characterize the intimate *X. fastidiosa*-*P. spumarius* interaction. First, possible
645 plant effects on the behavioral manipulation should be excluded by artificial acquisition of the
646 bacterium; second, observations should be extended to males and to the entire adult life span,
647 also increasing the duration of the IAP; third, it should be verified if such behavioral effect is
648 under genetic control of *X. fastidiosa*

649

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Author Contribution

- DC and AF conceived research.
- DC and MoM conducted experiments.
- DC, MaM and EG analyzed the data.
- DC wrote the manuscript.
- MoM, MaM, EG, AM, MS and AF reviewed and edited the manuscript.
- AM, MS and AF secured funding.
- All authors read and approved the manuscript.

Data availability statement

Additional data will be furnished by the authors upon reasonable request.

Competing interests statement

The authors declare no competing interests.

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918

919 **Tables**

920 **Tab.1 Acquisition behavior. Table "Acquisition" (on the left) summarizes the experimental design, the treatments, and the**
921 **number of replicates, together with the number of spittlebugs that acquired the bacterium for each treatment. The tables on the**
922 **right, report three EPG non-sequential variables (WDI, WDEI and NWEI) calculated for the two spittlebugs that acquired *X.***
923 ***fastidiosa*.** WDI: waveform duration per individual. WDEI: waveform duration per event per individual. NWEI: number of waveform
924 events per individual. The variables are described in Tab. 4. Time is expressed as minutes.

Acquisition		
Treatment	**n Replicates§**	**Ps positive§§**
C	30	0
Xc	34	0
1h	30	1
3h	37	1

WDI§§§								
AAP	**Succ pr**	**Unsucc pr**	**np**	**C**	**Xc**	**Xi**	**N**	**R**
1h	1	0	28.5	13.7	1.5	14.8	2.3	1.2
3h	1	3	24.2	27.6	4.4	122.8	0.2	0.8

WDEI§§§								
AAP	**Succ pr**	**Unsucc pr**	**np**	**C**	**Xc**	**Xi**	**N**	**R**
1h	1	0	14.5	4.56	1.5	4.93	0.19	1.2
3h	1	3	6.05	3.94	1.1	61.4	0.2	0.8

NWEI§§§								
AAP	**Succ pr**	**Unsucc pr**	**np**	**C**	**Xc**	**Xi**	**N**	**R**
1h	1	0	2	3	1	3	12	1
3h	1	3	4	7	4	2	1	1

§=number of replicates per each treatment; §§=number of spittlebugs that acquired *Xylella fastidiosa;*

§§§=WDI, WDEI and NWEI calculated only for the spittlebugs that acquired (and retained) the bacterium.

925

926 **Tab.2 Inoculation behavior.** "Sequence of events" stands for the sequence of behaviors performed by the insect before interrupting
927 the probe (not shown for the 1-hour and 3-hours IAP). "Ps positive" stands for the number of replicates carried out with infective
928 spittlebugs (as determined by qPCR) for each treatment. "Inoculation" indicates the number of plants inoculated with *X. fastidiosa*
929 by the qPCR positive *P. spumarius* per each treatment. NA (not applicable) is used for the sequence of events of the treatments 1h
930 and 3h, since the insects were given access to the plant without interrupting the probe after a precise event/sequence of events.

A)

Inoculation 2017 (olive)				
Treatment		**Sequence of events**	**Ps positive**	**Inoculation**
Waveform interruption	C	**C**	3	0
	Xc	**C-Xc**	9	0
	Xi	**C-Xc-Xi**	6	0
	N	**C-Xc-N or C-Xc-Xi-N§**	12	0
1h		**NA**	12	2

B)

Inoculation 2018 (olive)				
Treatment		**Sequence of events**	**Ps positive**	**Inoculation**
Waveform interruption	C	**C**	17	0
	Xc	**C-Xc**	16	0
	Xi	**C-Xc-Xi**	21	0
		C-Xc-Xi-N-Xi or C-Xc-N-Xc-Xi§§	15	0
	N	**C-Xc-N or C-Xc-Xi-N§**	30	0
	R	**C-Xc-Xi-R**	19	0
3h IAP§§§		**NA**	63§§§	3§§§

C)

Inoculation 2018 (oleander)				
Treatment		**Sequence of events**	**Ps positive**	**Inoculation**
Waveform interruption	C	**C**	8	0
	Xc	**C-Xc**	1	0
	Xi	**C-Xc-Xi**	8	0
	N	**C-Xc-Xi-N**	7	0
	R	**C-Xc-Xi-R**	1	0
	Xe	**C-Xc-Xe or C-Xc-Xi-Xe**	5	3

§=probe interrupted after the first N occurred; §§=the spittlebugs performed from 1 to 3 xylem interruptions N; §§§=calculated by pooling together the inoculation results from the 3h IAP inoculation experiment (49 infective spittlebugs) and the comparison infective vs non-infective (14 infective spittlebugs)

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933 **Tab.3 Comparison of infective versus non-infective *P. spumarius* probing behavior.** WDI: waveform duration per individual. WDEI:
934 average waveform duration per event per individual. NWEI: number of waveform events per individual. Sequential variables are
935 variables related to a succession of events/behaviors. The EPG variables are explained in Tab. 4. Time is expressed as minutes.

	WDI									
	Infective (n=14)				Non infective (n=35)				Mann-Whitney	
	min	max	mean	se (±)	min	max	mean	se	W	p
np*	12.3	79.4	47.36	6.15	0.7	114.8	32.06	5.19	340	0.036
C	1.1	47.2	11.16	4	1.1	33.7	8.26	1.29	228	0.707
Xc	0.1	7.3	2.914	0.51	0.4	11.9	2.72	0.4	281	0.425
Xi*	32.9	163.2	89.88	10.01	47.6	163.7	116	4.94	136	0.016
N	0	2.5	0.63	0.21	0	6.4	1.25	0.24	171	0.100
R	0	99.3	28.68	8.48	0	74.6	20.97	3.5	260.5	0.731

	WDEI									
	Infective (n=14)				Non infective (n=35)				Mann-Whitney	
	min	max	mean	se (±)	min	max	mean	se	W	p
np*	5.9	79.4	16.82	5.02	0.7	29.06	8.28	1.18	350	0.020
C	0.52	5.721	1.84	0.47	0.5	11.23	1.81	0.35	229.5	0.731
Xc	0.1	7.3	1.64	0.48	0.3	11.9	1.57	0.37	283	0.400
Xi	1.73	81.6	22.79	6.89	2.57	155.1	22.28	4.91	229	0.723
Xi<10min	1.73	6.13	3.91	0.42	0	11.22	3.43	0.37	294	0.278
Xi>10min	0	157.9	45.82	13.33	0	155.1	45.72	7.48	227	0.690
N	0	0.62	0.22	0.05	0	0.4	0.2137	0.018	241	0.928
R	0	14.8	3.55	1.04	0	11.4	2.47	0.4	282	0.412

	NWEI									
	Infective (n=14)				Non infective (n=35)				Mann-Whitney	
	min	max	mean	se (±)	min	max	mean	se	W	p
np	1	9	4.00	0.65	1	11	4.17	0.49	245.5	0.991
C	1	10	5.57	0.86	1	14	5.82	0.63	241	0.929
Xc	0	5	2.07	0.35	1	5	2.17	0.19	231	0.747
Xi	2	19	8.21	1.52	1	36	11.03	1.53	204	0.363
Xi<10min	1	19	6.50	1.48	0	36	8.43	1.58	234.5	0.815
Xi>10min*	0	4	1.71	0.28	0	6	2.6	0.22	152.5	0.032
N*	0	8	1.86	0.60	0	21	4.8	0.88	146	0.027
R	0	18	6.36	1.47	0	34	9.03	1.54	215	0.505

	SEQUENTIAL VARIABLES									
	Infective (n=14)				Non infective (n=35)				Mann-Whitney	
	min	max	mean	se (±)	min	max	mean	se	W	p
np to Xc	2	84	16.15	6.02	1.2	45.4	9.91	1.57	253.5	0.546
np to Xi	2.4	85.3	17.08	5.89	1.5	46.5	11.87	1.6	253.5	0.851
C to Xc	0.5	8.2	3.18	0.76	0.6	22.6	5.27	0.91	165.5	0.150
C to Xi	1	12.7	4.99	0.98	1.1	24.4	7.22	1.01	187	0.199
np to Xi>10	6.6	68	25.13	4.42	1.5	118	31.46	5.49	209.5	0.891
C to Xi>10	2.2	63.8	17.79	4.49	1.1	117.4	26.68	5.38	187	0.671
Time to the 1st probe*	0.5	79.4	12.08	5.60	0.05	43.7	4.65	1.47	346.5	0.024
Time to the 1st probe with Xi	1.11	79.4	13.98	5.61	0.4	44.66	8.08	1.58	209.5	0.314
Time to the 1st probe with Xi>10	1.11	66.2	17.13	5.06	0.4	110.3	16.52	4.13	238	0.395

	OTHERS VARIABLES									
	Infective (n=14)				Non infective (n=35)				Mann-Whitney	
	min	max	mean	se (±)	min	max	mean	se	W	p
Succ pr	1	5	2.00	0.28	1	5	2.05	0.18	238.5	0.879
Unsucc pr	0	7	1.78	0.57	0	10	2.03	0.41	234	0.799
Tot pr	1	8	3.78	0.64	1	11	4.08	0.48	239.5	0.902
Frequency Xi	2.78	6.68	4.40	0.30	0.23	0.65	0.43	0.01	247.5	0.956

*****= variables significantly different between infective and non-infective spittlebugs as indicated by Mann-Whitney U-test

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938

939 **Tab.4 Explanation of the meaning of the EPG variables calculated in the different experiments.** Frequency of the xylem ingestion
940 waveform (Xi) was calculated manually on intervals of 10 seconds; intervals were randomly selected every five minutes. The values
941 reported here are averages of all the intervals per each monitored spittlebug.

Variable abbreviation	**Variable definition**
Succ pr	number of successful probes (probes where the insect reaches the xylem)
Unsucc pr	number of unsuccessful probes (probes where the insect does not reach the xylem)
Tot pr	number of total probes (successful + unsuccessful)
np WDI	non-probing total duration per individual
C WDI	pathway total duration per individual
Xc WDI	xylem contact total duration per individual
Xi WDI	xylem ingestion total duration per individual
N WDI	xylem interruption total duration per individual
R WDI	resting total duration per individual
np NWEI	non probing total number of events per individual
C NWEI	pathway total number of events per individual
Xc NWEI	xylem contact total number of events per individual
Xi NWEI	xylem ingestion total number of events per individual
Xi<10min NWEI	xylem ingestion shorter than 10 minutes total number of events per individual
Xi>10min NWEI	xylem ingestion longer than 10 minutes total number of events per individual
N NWEI	xylem interruption total number of events per individual
R NWEI	resting total number of events per individual
np WDEI	average non probing duration of single events per individual
C WDEI	average pathway duration of single events per individual
Xc WDEI	average xylem contact duration of single events per individual
Xi WDEI	average xylem ingestion duration of single events per individual
Xi<10min WDEI	average xylem ingestion shorter than 10 minutes duration of single events per individual
Xi>10min WDEI	average xylem ingestion longer than 10 minutes duration of single events per individual
N WDEI	average xylem interruption duration of single events per individual
R WDEI	average resting duration of single events per individual
np to Xc	time from the beginning of the recording to the first xylem contact
np to Xi	time from the beginning of the recording to the first xylem ingestion
C to Xc	time from the first probe to the first xylem contact
C to Xi	time from the first probe to the first xylem ingestion
np to Xi10	time from the beginning of the recording to the start of the first xylem ingestion longer than 10 minutes
C to Xi10	time from the first absolute probe to the start of the first xylem ingestion longer than 10 minutes
Time to the 1st probe	time from the beginning of the recording to the first probe
Time to the 1st probe with Xi	time from the beginning of the recording to the first probe with a xylem ingestion
Time to the 1st probe with xi>10	time from the beginning of the recording to the first probe with a xylem ingestion longer than 10 minutes
Frequency Xi	average frequency of the peaks of the xylem ingestion waveform (Hz)

942

943 **Figures**

944 **Fig.1 EPG waveforms (behavioral patterns) displayed by *Philaenus spumarius*.** a) waveform C (pathway); b) waveform Xc (xylem
945 contact/trial ingestion); c) waveform Xi (xylem ingestion); d) waveform N (interruption during the xylem activity); e) waveform R
946 (resting phase); f) waveform Xe (spikelet burst). Time (sec) is reported on the x-axis; Voltage (V) is reported on the y-axis. Images a to
947 e are derived from EPG recordings made with *P. spumarius* on olive plants; image f is derived from a recording made with *P.*
948 *spumarius* on oleander.

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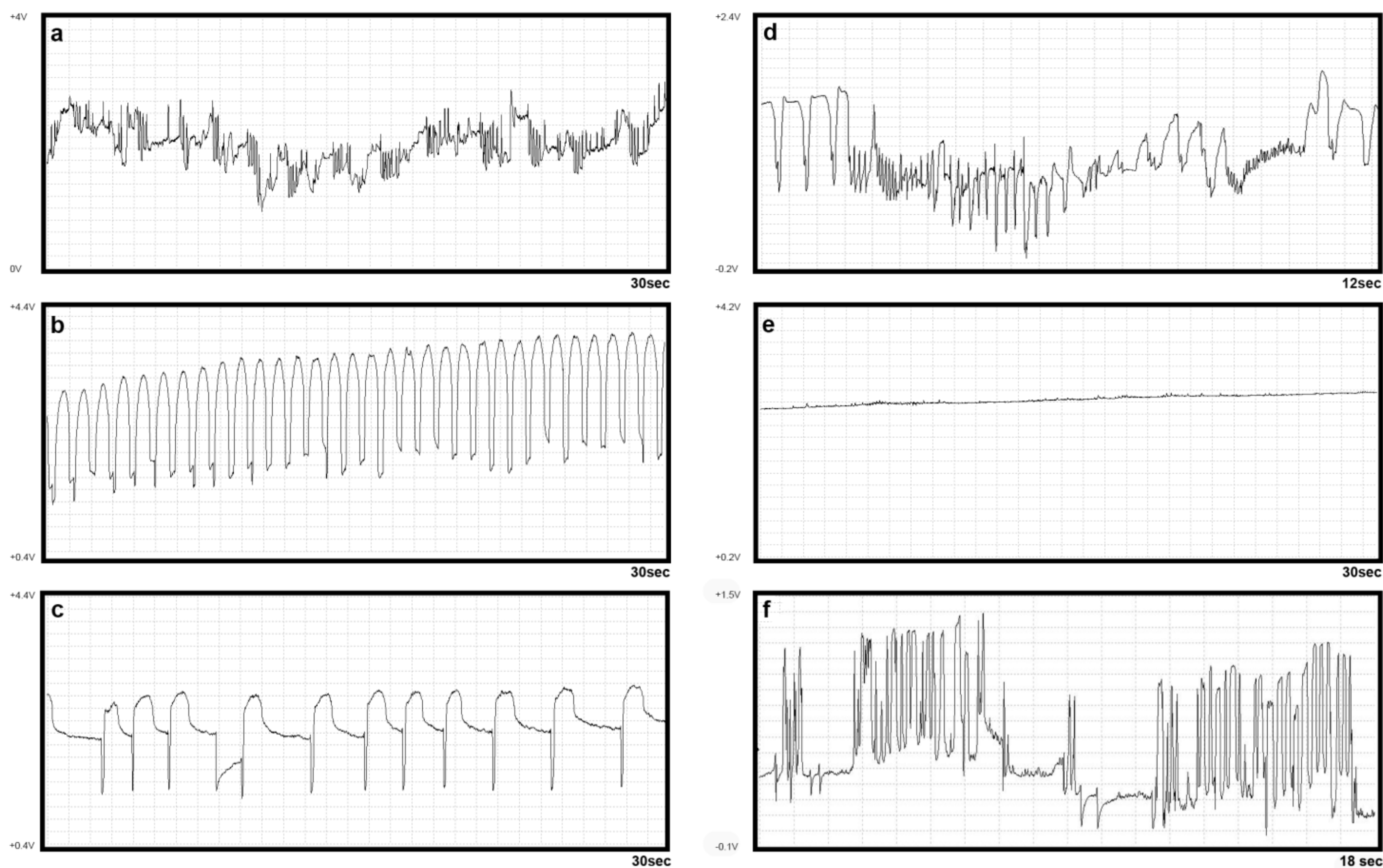

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951 **Fig.2 Xe waveform.** a and b) coarse structure of Xe (simple spikelet burst) in oleander following pathway C and xylem contact Xc,
952 coarse structure; c) coarse structure Xe (voltage drop) in olive following a resting phase alternated with very low frequency xylem
953 ingestion (≤0.1 Hz) Xi/R; d) fine structure of the spikelet burst in figure 2.a; e) fine structure of the spikelet burst in figure 2.b; f) fine
954 structure of the drop in figure 2.c. Spikelet bursts in figures 2.a, 2.b, and 2.c are indicated with black arrows. Time (sec) is reported
955 on the x-axis; Voltage (V) is reported on the y-axis.

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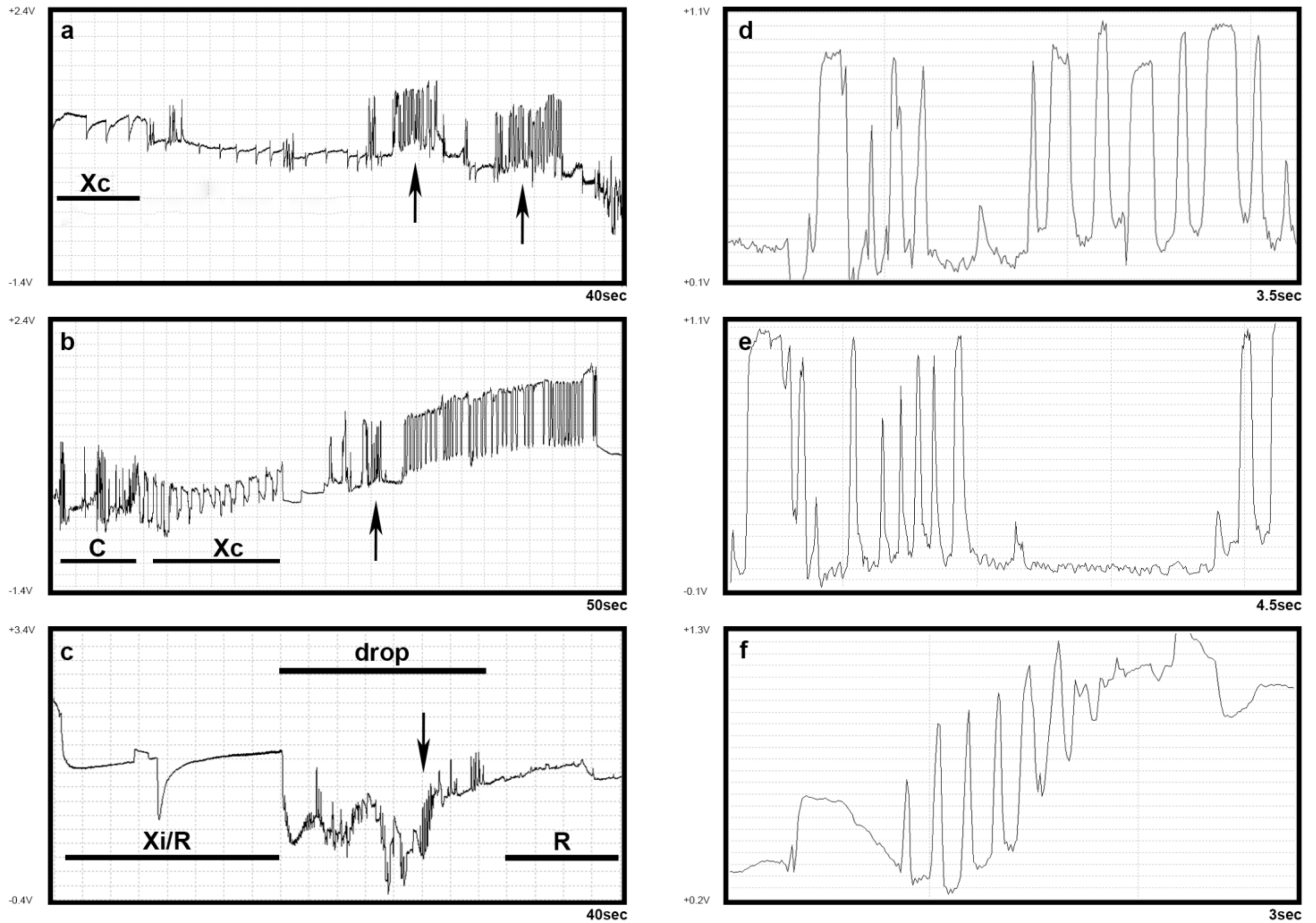

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